Biochem. J. (2002) 366, 745–755 (Printed in Great Britain)

P2X$_7$ receptors activate protein kinase D and p42/p44 mitogen-activated protein kinase (MAPK) downstream of protein kinase C

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Protein kinase D (PKD), also called protein kinase Cμ (PKCμ), is a serine/threonine kinase that has unique enzymic and structural properties distinct from members of the PKC family of proteins. In freshly isolated rat parotid acinar salivary cells, extracellular ATP rapidly increased the activity and phosphorylation of PKD. The stimulation by ATP required high concentrations, was mimicked by the P2X$_7$ receptor ligand BzATP [2′- and 3′-*O*-(4-benzoylbenzoyl)ATP], and was blocked by Mg^{2+} and 4,4′-di-isothiocyano-2,2′-stilbene disulphonate (DIDS), suggesting that activation of PKD was mediated by P2X$_7$ receptors, which are ligand-gated non-selective cation channels. Phorbol ester (PMA) and the activation of muscarinic and substance P receptors also increased PKD activity. PKC inhibitors blocked ligand-dependent PKD activation and phosphorylation, determined by *in vitro* phosphorylation studies and by phospho-specific antibodies to two activation loop sites (Ser^{744} and Ser^{748}) and an autophosphorylation site (Ser^{916}). ATP and BzATP also increased the tyrosine phosphorylation and activity of PKCδ, and these stimuli also increased extracellular signal-regulated protein kinase (ERK) 1/2 activity in a PKC-dependent manner. PKD activation was not promoted by pervanadate (an inhibitor of tyrosine phosphatases) and was not blocked by PP1 (an inhibitor of Src family kinases) or genistein (a tyrosine kinase inhibitor), suggesting that tyrosine kinases and phosphatases did not play a major role in PKD activation. P2X$_7$ receptor-mediated signalling events were not dependent on Ca^{2+} entry. These studies indicate that PKC is involved in cellular signalling initiated by P2X$_7$ receptors as well as by G-protein-coupled receptors, and demonstrate that PKD and ERK1/2 are activated in similar PKC-dependent signalling pathways initiated by these diverse receptor types.

Key words: muscarinic receptor, parotid acinar cell, PKCδ, salivary cell, signal transduction.

INTRODUCTION

Extracellular signals, including growth factors and neurotransmitters that activate plasma membrane receptors, lead to the rapid production of second messengers that can affect diverse cellular functions. Frequently these stimuli initiate multi-protein signalling cascades in response to the hydrolysis of lipids and/or the production of lipid second messengers. Among the signal-transduction proteins that play an integral part in many signalling cascades and which are activated by many different types of stimuli are protein kinases C (PKCs). Members of the PKC family of proteins are serine/threonine kinases that have been implicated in the regulation of physiological events such as ion and protein secretion. PKC proteins can also contribute to cell proliferation or differentiation through their participation in various signalling cascades, such as those that lead to activation of the mitogen-activated protein (MAP) kinases. The PKC family is subdivided into three subgroups. Members of the conventional PKC (cPKC) group include PKCα, βI, βII and γ, and are activated by Ca^{2+}, phospholipid and diacylglycerol (DAG). Members of the novel PKC (nPKC) group include PKCδ, ϵ, η and θ, and are activated by phospholipid and DAG but not by Ca^{2+}. The atypical group (PKCζ and λ/ι) are not regulated by Ca^{2+} or DAG, and their regulation may involve phosphoinositides [1]. PKC proteins can be activated by G-protein-coupled receptors (GPCRs), growth factors, cytokines and a variety of other stimuli [2–4].

Previously, we observed that PKCδ became phosphorylated on tyrosine residues that increased its activity when parotid acinar salivary gland cells were exposed to agonists of muscarinic and substance P receptors [5,6], which are both linked by heterotrimeric G-proteins to phospholipase C (PLC). Since neurotransmitters that bind to muscarinic and substance P receptors initiate fluid secretion by the parotid gland, which is one of the three main salivary glands, we hypothesized that PKCδ was involved in fluid and electrolyte secretion by these cells [5]. Ion-transport systems that participate in the initiation of fluid secretion by parotid acinar cells are activated not only by the M3 muscarinic receptor and other PLC-linked GPCRs but also by high concentrations of extracellular ATP acting on P2X$_7$ receptors. The similar effects of ATP and the muscarinic agonist carbachol on parotid ion-transport systems were due to their elevation of intracellular free calcium concentration ($[Ca^{2+}]_i$), albeit by different mechanisms [7,8].

Many cells have multiple extracellular nucleotide-binding P2 receptors, which are classified into two subfamilies, P2X and P2Y, each consisting of multiple members. P2Y receptors are metabotropic receptors that are coupled to G-proteins, and P2X receptors are ligand-gated ion channels [9–12]. Parotid acinar cells have two P2X receptors that are ligand-gated ion channels

Abbreviations used: BzATP, 2′- and 3′-*O*-(4-benzoylbenzoyl)ATP; $[Ca^{2-}]_i$, intracellular free calcium concentration; DAG, diacylglycerol; DIDS, 4,4′-di-isothiocyano-2,2′-stilbene disulphonate; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; GPCR, G-protein-coupled receptor; MAP, mitogen-activated protein; PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; PKD, protein kinase D; PLC, phospholipase C.

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and which are not coupled to heterotrimeric G-proteins [7,13–16]. These receptors can be distinguished by their different sensitivities to ATP: $P2X_4$ and $P2X_7$ receptors have a high and low affinity, respectively, for ATP [14,17]. The $P2X_7$ receptor was previously designated the P2Z receptor [18], and is found in macrophages, lymphocytes and other cells of immune lineage, in addition to salivary gland and other cells [16,19]. ATP increases $[Ca^{2+}]_i$ via the influx of Ca^{2+} into parotid acinar cells through these P2X channels [7,8,14,20–22], thus providing a mechanism of $[Ca^{2+}]_i$ elevation distinct from PLC-linked GPCRs.

Recently, several studies found that the activation of protein kinase D (PKD), a PKC-related protein, is dependent on PKC family members [23–25]. PKD, which is also called PKCμ, is a newly described serine/threonine protein kinase [26,27]. Compared to PKC proteins, PKD has several unique structural properties that indicate that it is in a separate class of proteins. PKD has a pleckstrin homology domain, an N-terminal hydrophobic domain, two cysteine-rich zinc finger domains and lacks the typical autoinhibitory pseudosubstrate domain of PKC. The optimum substrate specificity for PKD is distinct from that of PKC proteins [26–28]. In some studies, PKD has been localized to the Golgi apparatus [29], although it also has been found in the cytosol and other compartments [30].

Although muscarinic and $P2X_7$ receptors activate similar salivary ion-transport events that are initiated by the elevation of $[Ca^{2+}]_i$, we assumed that these two receptors would not activate many signalling proteins in common. However, recently the $P2X_7$ receptor was reported to form a signalling complex with 11 proteins, including a tyrosine phosphatase and a lipid kinase [31], suggesting that this ion channel may play a role in cell signalling involving tyrosine phosphorylation and lipid production. In preliminary studies we found that ATP and carbachol both activated PKD, and in subsequent experiments we found that the activation of $P2X_7$ receptors increased PKD activity in a PKC-dependent manner. This indicated that this ligand-gated ion channel stimulated PKC. Therefore, since we had already demonstrated that GPCRs activated PKCδ, we also examined the effects of $P2X_7$ ligands on PKCδ in parotid cells. In addition, we compared the effects of carbachol and $P2X_7$ agonists on another potential PKC-dependent event, the activation of the MAP kinases extracellular signal-regulated protein kinase (ERK) 1 and ERK2.

In addition to conducting *in vitro* assays to measure PKD enzyme activity and phosphorylation, we also used two phospho-specific antibodies to identify phosphorylation sites on PKD. One antibody recognizes an autophosphorylation site (Ser^{916}) that correlates with PKD enzyme activity when this residue is phosphorylated [32]. Another antibody recognizes two sites (Ser^{744} and Ser^{748}) on the activation loop that are phosphorylated in a PKC-dependent manner during PKD activation [33]. We also examined whether we could detect complexes of PKD with PKC proteins in parotid cells, since PKD can form complexes with PKC and other signalling proteins. Two members of the nPKC subgroup, PKCη and to a much lesser extent PKCϵ, co-associated with PKD in an immune complex when these proteins were overexpressed in COS-7 cells [34]. Moreover, the co-expression of PKD with either PKCη or PKCϵ produced a large increase in the phosphorylation and activity of PKD. The association and effects of these PKC proteins with PKD were dependent on the pleckstrin homology domain of PKD. The protein 14-3-3 [35] and the lipid kinases phosphatidylinositol 4-kinase and phosphatidylinositol-4-phosphate 5-kinase [36] also associate with PKD.

The present studies demonstrate that PKD, PKCδ and ERK1/2 are activated by both $P2X_7$ receptors and GPCRs, and demonstrate that PKC is upstream of both PKD and ERK1/2 activation by $P2X_7$ receptors in salivary gland epithelial cells. Thus ligand-gated $P2X_7$ receptors activate PKC and initiate multi-protein signalling pathways in common with those stimulated by PLC-linked GPCRs.

EXPERIMENTAL

Materials

All chemicals were reagent grade or better. Carbamyl choline (carbachol), orthovanadate and H_2O_2 were purchased from Sigma. Pervanadate was formed by combining H_2O_2 and vanadate in a 1:1 molar ratio. Substance P was obtained from Peninsula Laboratories. PMA was obtained from Life Technologies. Anti-PKD/PKCμ antibodies (SC-935, SC-638), anti-ERK2 antibody (SC-154) and polyclonal anti-PKCδ antibody (SC-213) were purchased from Santa Cruz Biotechnology. Monoclonal PKCδ was purchased from Transduction Laboratories. Phospho-ERK1/2 antibody and phospho-PKD antibodies (S916 and S744/S748) were obtained from Cell Signalling. Anti-phosphotyrosine antibody was a generous gift from Dr Thomas Roberts (Dana Farber Cancer Institute, Boston, MA, U.S.A.). Goat anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated antibodies were purchased from Chemicon. Synthetic peptides based on the predicted optimal substrates of PKC-βI, PKCδ and PKD [28] were a generous gift of Dr Lewis C. Cantley (Beth Israel Deaconess Medical Center, Boston, MA, U.S.A.). The ϵ peptide based on the pseudosubstrate domain of PKCϵ (PRKRQGSVRRRV) was obtained from Upstate Biotechnology. P81 phosphocellulose paper was purchased from Whatman. 4,4′-Di-isothiocyano-2,2′-stilbene disulphonate (DIDS) was purchased from Molecular Probes. PP1 [4-amino-5-(4-methylphenyl)-7(t-butyl)pyrazolo[3,4-d]pyrimidine], genistein, Ro31-8220 (3-{1-[3-(amidinothio)propyl]-1H-indol-3yl}-3-(1-methyl-1H-indol-3-yl)maleimide methane sulphate) and GF109203X {bisindolylmaleimide I; 2-[1-(3-dimethylaminopropyl)-1H-indol-3yl]-3-(1H-indol-3-yl)-maleimide} were purchased from Calbiochem. Male Sprague–Dawley rats (175–225 g) were obtained from Charles River Laboratories or Taconic.

Cell preparation and solutions

Freshly dispersed rat parotid acinar cells were prepared as described previously [20]. Usually the cells were suspended in a medium (solution A) composed of the following: 116.4 mM NaCl, 5.4 mM KCl, 1 mM NaH_2PO_4, 25 mM Na-Hepes, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM sodium butyrate and 5.6 mM glucose, pH 7.4. In experiments in which the effects of ATP and other nucleotides were examined, including those in which the effects of nucleotides and carbachol were examined using the same preparation of cells, solution A was modified to contain 1 mM $CaCl_2$ and no $MgCl_2$ was added. Cells were maintained on ice prior to use. Samples of the cell suspension were stirred and equilibrated at 37 °C for at least 10 min prior to use. In some experiments, cells were pre-treated with PP1 (10 μM), GF109203X (3 μM), Ro31-8220 (3 μM), genistein (100 μM), DIDS (100 μM) or vehicle (0.1% DMSO) for 15–20 min prior to the addition of stimuli.

Measurement of PKD enzyme activity and *in vitro* autophosphorylation

Cells were treated for the designated times and conditions and were collected by a brief spin in a microcentrifuge. The supernatant was removed, 1 ml of ice cold lysis buffer was added, and the lysates were vortexed and spun at 16000 *g* for 15 min at

4 °C. The lysis buffer [137 mM NaCl, 20 mM Tris (pH 7.5), 1 mM EGTA, 1 mM EDTA, 10 % (v/v) glycerol and 1 % (v/v) Nonidet P-40] contained the following phosphatase and protease inhibitors: 1 mM vanadate, 4.5 mM sodium pyrophosphate, 47.6 mM NaF, 9.26 mM β-glycerophosphate, 0.5 mM dithiothreitol, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin and 2 μg/ml 4-(2-aminoethyl)benzenesulphonylfluoride ('AEBSF'). The cleared supernatants were transferred to fresh 1.5 ml microcentrifuge tubes and incubated overnight with anti-PKD antibody at 1–1.5 μg/ml. Proteins were collected by centrifugation after a 1 h exposure to Protein A–Sepharose beads (4 mg). The immunoprecipitates were washed two times in ice-cold PBS/Nonidet P-40 (137 mM NaCl, 15.7 mM NaH_2PO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl and 1 % Nonidet P-40, pH 7.4), once in 0.1 M Tris (pH 7.5)/0.5 M LiCl and two times in PBS. All wash solutions except PBS contained 0.2 mM vanadate. The immunoprecipitated proteins were exposed to a reaction buffer consisting of the following: 5 mM $MgCl_2$, 0.5 mM EGTA, 100 μM PKC-βI synthetic substrate peptide or 25 μM PKCϵ synthetic peptide, 1 mM dithiothreitol, 25 mM Tris (pH 7.5), 20 μg/ml phosphatidylserine and 10 μM DAG. After a 10 min pre-equilibration period at 30 °C, the assay was initiated with the addition of ATP (50 μM ATP final concentration, 2 μCi [^{32}P]ATP). The total reaction mixture was 100 μl. In some experiments, GF109203X and DMSO were added to the immunoprecipitates during the pre-equilibration period prior to the addition of ATP. The samples were incubated at 30 °C for 30 min with intermittent mixing, and then duplicate 10 μl samples of each reaction mixture were spotted on to p81 phosphocellulose paper. Phosphorylation of the peptide substrate was linear over the 30 min period. Background activity was measured in 10 μl samples of the same reaction mixture that was added to Protein A–Sepharose beads. The p81 papers were washed five times in 0.425 % phosphoric acid, and the incorporation of ^{32}P on to p81 paper was determined by liquid scintillation counting. The duplicate values from each immunoprecipitate were averaged and treated as one sample. Usually two or more samples (separate immunoprecipitates) were collected for each of the various conditions in each experiment, and the multiple samples for each condition were averaged and analysed as the results from one experiment ($n = 1$ cell preparation). In each experiment, data were normalized to values obtained under basal (unstimulated) conditions for control (no inhibitor) cells.

In preliminary experiments (results not shown), PKD activity also was measured in anti-PKD immunoprecipitates using a synthetic peptide based on the predicted optimum substrate for PKCμ/PKD [28]. The results using the two different peptides (PKD and PKC-βI) were similar, as reported in other observations [36]. However, because the PKD peptide was slightly hydrophobic, we found that the assays conducted using the PKC-βI peptide were more linear over the 30 min assay period, so we used this peptide for the experiments reported here. Also, since an anti-PKD antibody (SC-935) based on a C-terminal peptide was more efficient in immunoprecipitating PKD activity than an antibody (SC-638) raised to a peptide based on the N-terminus, the C-terminus-based antibody was used for all experiments.

For *in vitro* phosphorylation studies, PKD was immunoprecipitated using the anti-PKD antibody and washed as described above. The immunoprecipitates were exposed to a reaction buffer consisting of the following (final concentrations): 30 mM Tris (pH 7.5), 1 mM dithiothreitol and 10 mM $MgCl_2$. After a 10 min pre-equilibration period at 30 °C, the assay was initiated with the addition of ATP (50 μM ATP final concentration/1 μCi of [^{32}P]ATP). The total reaction mixture was 100 μl. After 20 min of exposure to [^{32}P]ATP, the reaction was stopped by the addition of 1 ml of PBS containing 100 mM EDTA. The immunoprecipitates were collected by centrifugation, the supernatants were removed and replaced with 2 × Laemmli sample buffer, and boiled. The proteins were subjected to SDS/PAGE (3 % stacking gel, 7 % separating gel). The gels were dried and the phosphorylation of PKD was quantified using a Bio-Rad GS-525 Molecular Imager System.

PKC inhibitors and other agents were added to cells that subsequently were exposed to stimuli or vehicle (basal). The effects of each inhibitor were calculated by comparing the difference between the stimulated (A) and basal (B) PKD activities (or phosphorylations) in the presence of the inhibitor compared with the difference between the stimulated (C) and basal (D) PKD activities (or phosphorylations) in the absence of the inhibitor using the following formula: $100 \times (A-B)/(C-D)$. In general, the inhibitors or altered conditions (e.g. the removal of extracellular Ca^{2+}) did not produce large changes in the basal PKD activity (see Results).

Detection of PKD autophosphorylation and PKC-dependent PKD phosphorylation using phospho-specific antibodies

In some experiments, cell lysates were immunoblotted using phospho-specific antibodies to Ser^{916} and Ser^{744}/Ser^{748}, important sites for PKD activation (see Results). Parotid cells were treated with various stimuli and inhibitors as described above. Cells were rapidly collected by a brief spin in a microfuge, lysed in the standard lysis buffer and the membrane-cleared lysates were boiled in Laemmli sample buffer. Samples were subjected to SDS/PAGE using an 8 % acrylamide separating gel and a 3 % acrylamide stacking gel. Proteins were transferred to nitrocellulose paper, and blocked in 5 % non-fat dry milk in TTBS (20 mM Tris, pH 7.6, 137 mM NaCl and 0.2 % Tween 20). Immunoblots were sequentially probed overnight with the phospho-specific antibodies (1 : 1000 dilution) followed by a 1 h exposure to goat anti-rabbit horseradish peroxidase-conjugated antibody (1 : 50000 dilution). Proteins were visualized using a chemiluminescence system and X-ray film. The immunoblots were stripped and reprobed with anti-PKD antibody (SC-935 at 0.2 μg/ml).

Immunoprecipitations and Western blotting of PKCδ

Cell lysates cleared of membranes were obtained from parotid acinar cells as described above. For immunoprecipitations, the lysates were incubated with monoclonal PKCδ antibody ($\approx$ 0.5 μg/ml) for 3 h at 4 °C, after which Protein A–Sepharose (4 mg) was added for an additional 1 h. At the end of this period, proteins were collected by sedimentation, and the immunoprecipitates were washed three times in PBS/1 % Igepal. The pelleted proteins were diluted with 2 × Laemmli sample buffer, boiled for 4 min and stored at −20 °C prior to electrophoresis. Samples were separated using SDS/PAGE with an 8 % acrylamide separating gel and a 3 % stacking gel. Proteins were transferred to nitrocellulose paper, and blocked in 2 % BSA in TTBS. Immunoblots were sequentially probed overnight with monoclonal anti-phosphotyrosine antibody (1 μg/ml) and polyclonal anti-PKCδ antibody (0.5 μg/ml). Proteins were visualized using a chemiluminescence system and X-ray film.

PKCδ activity

PKCδ was immunoprecipitated as described above, and a phosphorylation assay was performed in the absence of exogenous lipid using a synthetic substrate peptide based on the optimum

phosphorylation sequence of PKCδ [28], as reported previously [6,37].

Activation of MAP kinases (ERK1/2)

Parotid cell lysates cleared of membranes were diluted 1:1 with 2 × Laemmli sample buffer, boiled and subjected to SDS/PAGE using a 10% separating gel and a 3% stacking gel. The proteins were transferred to nitrocellulose paper, blocked in TTBS plus 5% non-fat dry milk solution, and sequentially probed overnight with phospho-ERK1/2 (1:1000 dilution) and anti-ERK2 (0.1 μg/ml) antibodies.

Data

The numbers (n) of independent experiments are as noted on the figures or in the text. The means ± S.E.M. from n separate experiments (each obtained from a separate cell preparation) are as indicated. Differences between control and experimental samples for the accumulated data were evaluated using a two-tailed Student's t test. All immunoblots are representative of at least three separate experiments.

RESULTS

$P2X_7$ receptors activate PKD

In initial studies we found that ATP increased PKD activity. Parotid acinar cells have both high-affinity $P2X_4$ and low-affinity $P2X_7$ receptors that are activated by extracellular nucleotides. These P2X receptors are ion channels that are not directly coupled to heterotrimeric G-proteins. Since the activation of the $P2X_7$ receptor is greater in the absence of divalent cations, the experiments in which we examined the effects of ATP were performed in the absence of extracellular Mg^{2+}.

ATP (1 mM) promoted a time-dependent increase in PKD activity to a maximum level that was about twice that of the basal activity (Figure 1, top panel). To determine which receptor subtype activated PKD, we examined the concentration dependence of extracellular ATP on PKD activity. ATP (1 min) activated PKD in a concentration-dependent manner (Figure 1, second panel). A 1 mM concentration of ATP increased PKD activity to a much greater level than lower ATP concentrations. This suggested that the effects of ATP were mediated mainly by the low-affinity $P2X_7$ receptor. Several additional findings also indicated that the effects of 1 mM ATP were mediated by the $P2X_7$ receptor. Mg^{2+} (5 mM), which greatly reduces the effect of ATP on the $P2X_7$ receptor but not on the $P2X_4$ receptor in parotid cells [14,17], blocked ≈ 70% of the ATP-stimulated increase in PKD activity (Table 1). In addition, pretreatment of the cells with DIDS, which blocks effects of ATP on the low-affinity ($P2X_7$) receptor but not on the high-affinity ($P2X_4$) receptor [14,38], also blocked ≈ 70% of the increase in PKD produced by 1 mM ATP (Table 1). We also compared the effects of several other nucleotides on PKD activation. The increase in PKD activity produced by 2′- and 3′-*O*-(4-benzoylbenzoyl)ATP (BzATP; 100 μM), which preferentially activates the parotid $P2X_7$ receptor [14,17], was 107.4 ± 28.6% ($n = 3$) of the increase produced by ATP (1 mM). Thus, BzATP activated PKD to a level comparable to that produced by ATP. In contrast, UTP (1 mM), which preferentially activates several G-protein-coupled P2Y receptors, increased PKD activity by only 36.0 ± 8.3% ($n = 4$) of the increase produced by ATP (1 mM). Therefore, both the inhibitor data and the pharmacological nucleotide profile indicate that the stimulatory effects of ATP were primarily due to the activation of the low-affinity $P2X_7$ receptor.

PKD is activated by PLC-linked GPCRs but not by growth factor receptors

To more fully characterize PKD activation in parotid cells, we also measured the effects of other stimuli on PKD. Carbachol (100 μM), which activates the parotid M3 muscarinic receptor, promoted a very rapid increase in PKD activity (Figure 1, third panel). The maximum level reached was about twice the basal activity, similar to the increase produced by 1 mM ATP. Carbachol and ATP did not have additive effects on PKD activity. The increase in PKD activity by carbachol (100 μM) alone and by ATP (1 mM) alone were 91.2 ± 24.3% ($n = 3$) and 93.1 ± 8.0% ($n = 3$), respectively, of the increase produced by exposure of the cells to both stimuli combined (1 min exposure for all conditions).

Exposure of cells to substance P (100 nM, 1 min) and the phorbol ester PMA (100 nM, 5 min) increased PKD activity to about the same level as did ATP and carbachol (Figure 1, bottom panel). The M3 muscarinic receptor and the substance P receptor are both coupled to PLCβ by G_q. Parotid acinar cells also have β-adrenergic receptors, which are linked to adenylate cyclase by G_i. Isoproterenol, which activates parotid β-adrenergic receptors and which promotes the exocytosis of secretory granules containing amylase and other proteins, did not activate PKD (Figure 1, bottom panel). The growth factors insulin and epidermal growth factor (EGF) also did not produce a substantial increase in PKD activity. Ionomycin, a calcium ionophore, produced only a modest (≈ 20%) increase in PKD activity. Pervanadate, an inhibitor of protein tyrosine phosphatases, which greatly increases the overall degree of tyrosine phosphorylation in parotid acinar cells (results not shown), also did not produce a large change in PKD activity. These results indicate that growth factor receptor activation, general increases in tyrosine phosphorylation or cAMP and the elevation of $[Ca^{2+}]_i$ were insufficient to activate PKD in parotid acinar cells, but agonists to G_q-coupled receptors and $P2X_7$ receptors were effective stimuli.

Stimuli that increase PKD activity also promote PKD autophosphorylation

In other experiments, changes in *in vitro* PKD autophosphorylation levels were examined using PKD immunoprecipitated from cells exposed to various stimuli (Figure 2, top panel). Exposure of parotid cells to high concentrations of ATP promoted a rapid increase in PKD phosphorylation. Exposure of cells to carbachol, substance P and PMA also stimulated an increase in PKD autophosphorylation under these conditions. The increases in autophosphorylation were similar to the increases in PKD activity produced by these stimuli (Figure 2, bottom panel).

PKC inhibitors block the activation of PKD

Since several investigators reported that the activation of PKD was dependent on PKC activity, we examined the effects of exposing cells to inhibitors of PKC prior to treatment with stimuli. GF109203X blocked most of the activation of PKD by ATP and BzATP (Table 1). These results suggest that PKC is upstream of the stimulation of PKD by $P2X_7$ receptors. This was a surprising finding, since the activation of this receptor/ion channel is not usually considered to activate PKC. Two different inhibitors of PKC, Ro31-8220 and GF109203X, blocked most of the stimulatory effect of carbachol on PKD activity (Table 1). GF109203X (3 μM) also blocked most (72.2%, $n = 1$) of the PMA-stimulated increase in PKD activity. GF109203X also

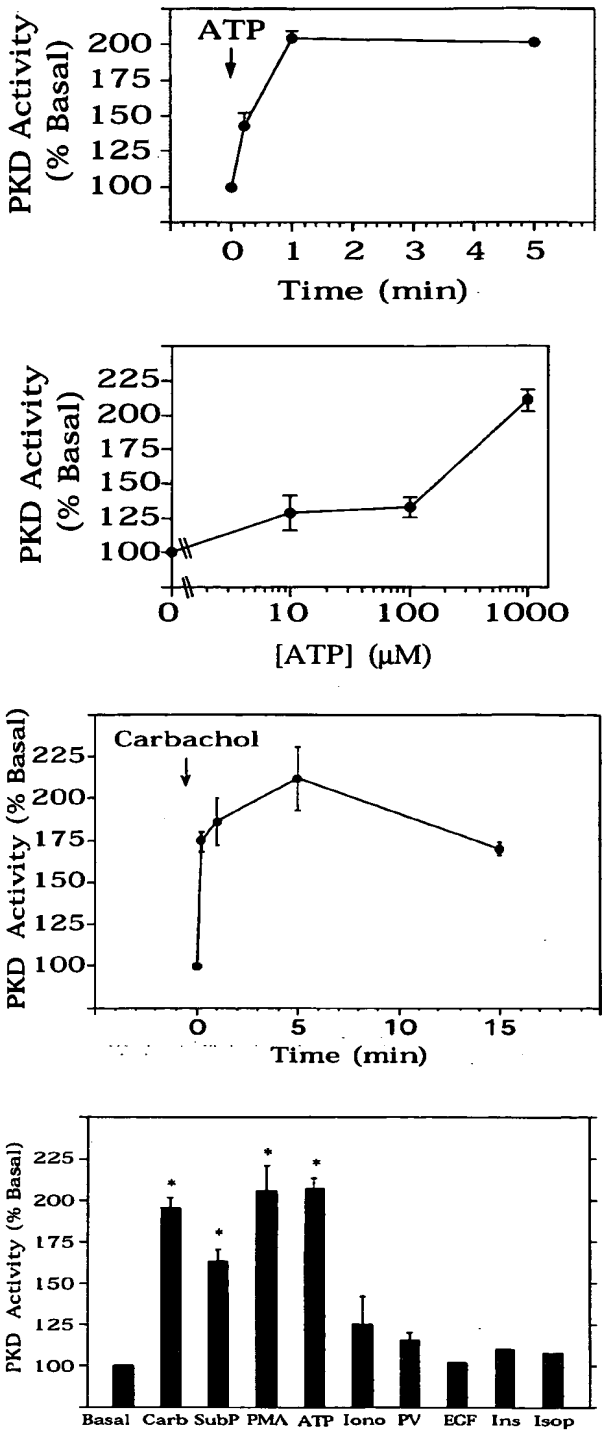

Figure 1 Effects of various stimuli on PKD activity

PKD activity in parotid acinar cells was measured in anti-PKD immunoprecipitates using a substrate-phosphorylation assay (see the Experimental section). The activities are shown relative to the activity in unstimulated (basal) cells. Top panel: time course of activation of PKD by ATP. Cells were exposed to ATP (1 mM) for 15 s ($n = 4$), 1 min ($n = 4$) or 5 min ($n = 2$). Second panel: concentration-dependence of ATP on PKD activity. Cells were exposed to 10 μM ATP ($n = 3$), 100 μM ATP ($n = 3$) or 1 mM ATP ($n = 4$) for 1 min. Third panel: time course of the activation of PKD by carbachol. Cells were exposed to carbachol (100 μM) for 15 s ($n = 3$), 1 min ($n = 4$), 5 min ($n = 4$) and 15 min ($n = 3$). Bottom panel: comparison of various agents on PKD activity. Cells were exposed to all stimuli for 1 min, except PMA (5 min). The following concentrations of stimuli were used: carbachol, 100 μM (Carb; $n = 29$); substance P, 100 nM (SubP; $n = 4$); PMA, 100 nM ($n = 12$); ATP, 1 mM ($n = 21$); ionomycin, 1 μM (Iono; $n = 6$); pervanadate, 100 μM (PV; $n = 3$); EGF, 100 ng/ml ($n = 2$); insulin, 100 nM (Ins; $n = 2$); isoproterenol, 100 μM (Isop; $n = 1$). *Significantly different ($P \leq 0.001$) from basal activity.

Table 1 Effects of inhibitors on basal and stimulated PKD activities in rat parotid acinar cells

Cells in suspension were treated with vehicle (0.1 % DMSO or water) or inhibitors for 15–20 min prior to the addition of carbachol (100 μM), ATP (1 mM), BzATP (10 μM) or vehicle (water) for 1 min. PKD activity was measured in anti-PKD immunoprecipitates as described in the Experimental section. The following concentrations of inhibitors were used: 3 μM Ro31-8220, 3 μM GF109203X, 10 μM PP1, 100 μM genistein, 5 mM Mg^{2-} and 100 μM DIDS. Extracellular Mg^{2-} was normally absent in experiments in which the effects of ATP or BzATP were examined. The effects of the inhibitors on the basal PKD activity (column 2) were determined by comparison with the basal (unstimulated) activity in PKD immunoprecipitated from cells not treated with inhibitors. To calculate the normalized increases in PKD activity by the different stimuli (columns 3 and 4), the appropriate basal values (±inhibitor) were subtracted, and the stimulus-dependent increases in PKD in inhibitor-treated cells were compared to those of control (no inhibitor) cells exposed to the same stimuli (see the Experimental section). The numbers shown in parentheses are the numbers of experiments. N.D., not determined. *Significantly different ($P \leq 0.002$) from control increase. **Significantly different ($P \leq 0.01$) from control basal.

Inhibitor	Basal PKD activity (% of control activity)	Increase in PKD activity (% of control increase)	
		Carbachol	ATP
Control (vehicle)	100%	100%	100%
Ro31-8220	85.8 ± 3.2%** (3)	38.1 ± 8.7%* (3)	N.D.
GF109203X	84.9 ± 3.1%** (9)	31.5 ± 6.1%* (6)	1.7 ± 1.0%* (3) [with BzATP, 24.2 ± 3.4%* (3)]
PP1	96.0 ± 2.7% (6)	120.5 ± 22.9% (6)	86.0 ± 11.9%* (3)
Genistein	89.4 ± 10.3% (3)	94.5 ± 9.0% (3)	N.D.
DIDS	83.8 ± 10.8% (3)	N.D.	31.5 ± 9.0%* (3)
Mg^{2-}	92.3 ± 3.2% (4)	N.D.	29.8 ± 6.8%* (4)

reduced the carbachol-promoted increase in PKD phosphorylation (Figure 2, top panel) by 55.7 ± 6.2% ($n = 3$). Ro31-8220 and GF109203X inhibit members of the cPKC and nPKC subgroups, suggesting that one or more of these PKC family members contribute to the PKD activation by these stimuli.

Since the conclusion that PKD activation depends on PKC is based, in part, on the specificity of GF109203X as an inhibitor of PKC, we performed several additional experiments. The concentration of GF109203X used in these studies had been chosen because it blocked > 95% of the activity of PKCδ *in vitro* [37]. To rule out a non-specific effect of GF109203X directly on the ATP-binding $P2X_7$ receptor itself, we examined the effect of GF109203X (3 μM) on ATP-promoted increases in $[Ca^{2+}]_i$ in Fura-2-loaded cells as an indication of $P2X_7$ receptor activation. The elevation of $[Ca^{2+}]_i$ by ATP (100 and 1000 μM) was not blocked in the presence of this PKC inhibitor (results not shown). To rule out a direct effect of GF109203X on PKD itself, we determined whether GF109203X directly inhibited PKD activity by adding either GF109203X (3 μM) or vehicle (0.06% DMSO) *in vitro* to PKD immunoprecipitated from both unstimulated and PMA-treated cells. The basal activity in the presence of GF109203X was 82.1 ± 3.2% ($n = 4$) of the control (vehicle) basal activity. The increase in PKD activity observed in PMA

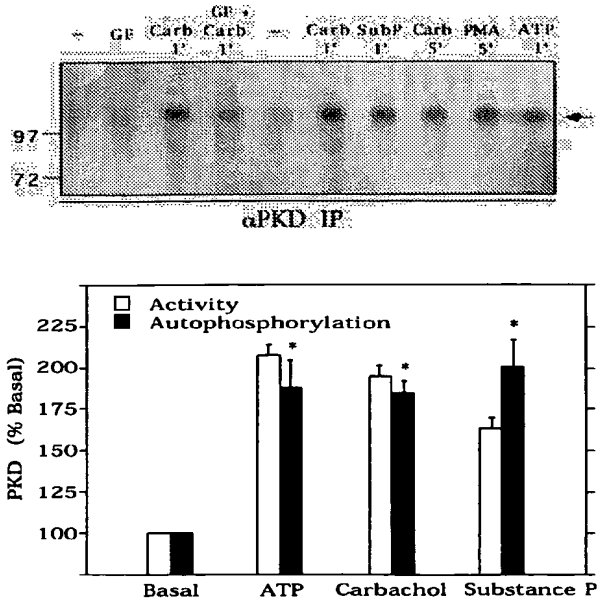

Figure 2 Effect of various stimuli on the phosphorylation of PKD

PKD was immunoprecipitated from cells subjected to various conditions, and the immunoprecipitates were subjected to an *in vitro* autophosphorylation assay (see the Experimental section). The immunoprecipitated proteins were then subjected to SDS/PAGE, and the dried gels were imaged and quantified. Top panel: autoradiogram of the phosphorylation of PKD (arrow) immunoprecipitated from cells treated with various agents. Bottom panel: quantification of the alterations in PKD phosphorylation (filled bars) relative to unstimulated (basal) levels. The effects of the stimuli on PKD activity (open bars) reproduced from Figure 1 (bottom panel) are shown for comparison. The stimuli (and number of phosphorylation experiments) used here were: carbachol, 100 μM (Carb; $n = 4$); Substance P, 100 nM (SubP; $n = 3$); and ATP, 1 mM ($n = 3$). When added, GF109203X (GF; 3 μM) was present for 15 min prior to stimulation. *Significantly different ($P \leq 0.001$) from basal activity.

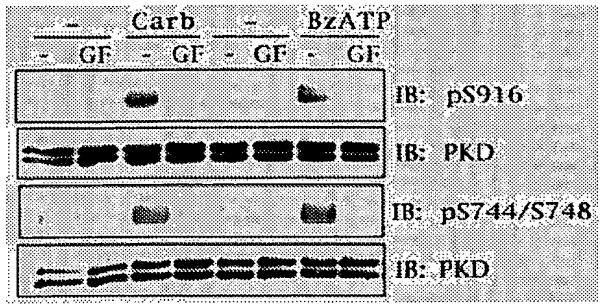

Figure 3 BzATP and carbachol increase PKD phosphorylation at the autophosphorylation site Ser^{916} and transphosphorylation sites Ser^{744}/Ser^{748}

The phosphorylation status of PKD in parotid acinar cells was identified by immunoblotting lysates with two phospho-specific antibodies. Cells were exposed to carbachol (10 μM) or BzATP (10 μM) for 2 min after pre-treatment with vehicle (0.1 % DMSO) or 3 μM GF109203X (GF) for 15–20 min prior to stimulation. Lysates were subjected to SDS/PAGE, transferred to nitrocellulose blotting paper and sequentially immunoblotted (IB) using different phospho-specific antibodies to detect increases in phosphorylation on different sites on PKD. The blots were stripped of antibody and reprobed with a PKD-specific antibody. The upper two blots and lower two blots were from identical samples run on two different gels. The major phosphorylated bands co-migrated with the upper bands of the doublet identified in the α-PKD immunoblot. Similar results were obtained in at least two other experiments.

(100 nM)-treated cells (i.e. the difference between the stimulated and basal activities) was 84.3 ± 12.6 % ($n = 4$) in the presence of GF109203X compared with vehicle alone. Thus, although there may be a modest effect of GF109203X on PKD activity under our assay conditions, the large ($\approx$ 70–100 %) reductions in the stimulus-dependent PKD activation in GF109203X-treated cells are not explained by a direct inhibition of PKD. Other investigators used GF109203X and Ro31-8220 to block the activation of PKD by PDGF and other stimuli, and also reported that these compounds did not directly block PKD enzymic activity [23–25, 39]. Note also that when we evaluated the effects of inhibitors added to intact cells, we always measured their effects on both basal and activated PKD activities in order to calculate the net effects of the inhibitors on stimuli-dependent increases in activity (see the Experimental section). None of the inhibitors, including the PKC inhibitors Ro31-8220 and GF109203X, that we tested on intact cells produced large alterations in the basal activity of PKD (Table 1). From the results shown in Table 1 and from these controls, we concluded that carbachol and ATP/BzATP activated a PKC-dependent pathway that was upstream of PKD.

PKC-dependent phosphorylation and activation of PKD determined using phospho-specific antibodies

We also examined PKD phosphorylation using two phospho-specific antibodies. One antibody recognizes phosphorylated Ser^{916}, an autophosphorylation site that is phosphorylated when PKD is activated. Another antibody recognizes the phosphorylation of Ser^{744} and Ser^{748}, PKD residues that are transphosphorylated in a PKC-dependent manner. The phosphorylation of PKD at these sites was increased in parotid cells exposed to BzATP and carbachol, and these phosphorylations were blocked in cells treated with the PKC inhibitor GF109203X (Figure 3). Substance P and PMA also increased phosphorylation at these sites (results not shown). These results complement those obtained when PKD enzyme activity was measured using a substrate phosphorylation assay (Figure 1) and when PKD phosphorylation was measured *in vitro* (Figure 2). Thus, these studies indicate that PKD activation and phosphorylation are dependent on PKC upon activation of the $P2X_7$ and other receptors in parotid cells.

Lack of association of PKC isoforms with PKD

PKD can form a complex with several nPKC proteins and can be co-immunoprecipitated with these PKCs, particularly PKCη. This association was detected in anti-PKD immunoprecipitates when *in vitro* substrate-phosphorylation assays were conducted using a substrate peptide (ϵ) that is suitable for nPKC proteins and other PKC proteins but which is a poor substrate for PKD [34]. We used this technique to determine whether a similar association between PKD and a member of the PKC family of proteins could be detected in rat parotid acinar cells under basal conditions or in cells exposed to stimuli (Figure 4). There was a robust basal PKD activity (typically $\approx$ 10000–30000 c.p.m.) in the *in vitro* substrate-phosphorylation assay conducted using anti-PKD immunoprecipitates that were assayed using the usual PKD-compatible substrate peptide (βI), and the activity was increased in immunoprecipitates from cells exposed to stimuli. In contrast, there was only a very small activity in anti-PKD immunoprecipitates that were assayed using a peptide (ϵ) that can be phosphorylated by PKCη, PKCϵ, PKCδ and other PKC proteins, but which is phosphorylated extremely poorly by PKD

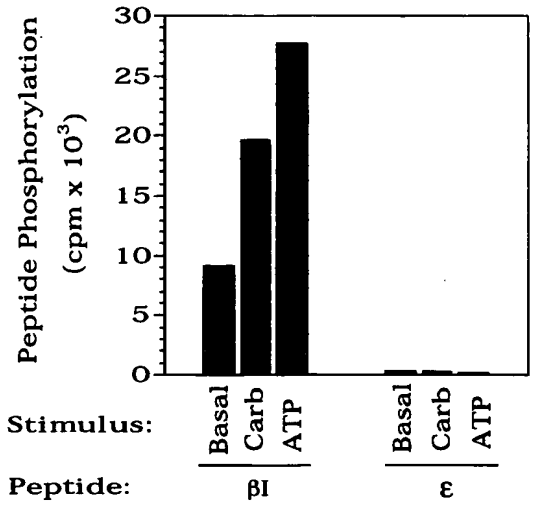

Figure 4 ***In vitro*** **phosphorylation of two different substrate peptides by anti-PKD immunoprecipitates from cells exposed to carbachol and ATP**

PKD was immunoprecipitated from unstimulated cells (Basal) or cells exposed for 1 min to 100 μM carbachol (Carb) or 1 mM ATP. The immunoprecipitates were subjected to an *in vitro* substrate-phosphorylation assay using either βI peptide (a good substrate for PKD) or ϵ peptide (a poor PKD substrate, but a good cPKC and nPKC substrate). When the βI peptide was used, significant substrate phosphorylation was observed under basal conditions, and the activity increased in immunoprecipitates from cells exposed to carbachol and ATP, as shown in other figures. There was a small but detectable phosphorylation of the ϵ peptide under the same conditions, but the activity did not change in agonist-treated cells. Similar results were obtained in two other experiments.

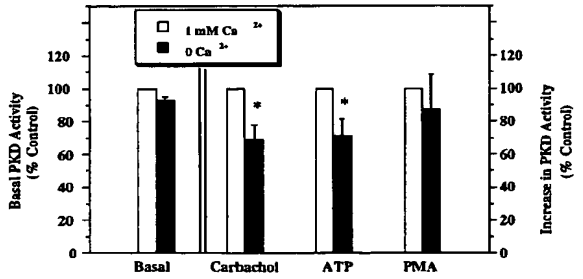

Figure 5 Contribution of extracellular Ca^{2+} to the activation of PKD

PKD activity in anti-PKD immunoprecipitates was measured using a substrate-phosphorylation assay (see the Experimental section). Cells were suspended in the presence of 1 mM Ca^{2-} (control conditions) or in the absence of Ca^{2-} plus the addition of 100 μM EGTA (0 Ca^{2-}). Changes in basal PKD activity (bars marked Basal) correspond to the left-hand axis, and changes in stimulus-dependent increases in PKD activity (right-hand sets of bars) correspond to the right-hand axis. The following concentrations of stimuli were used: carbachol, 100 μM; ATP, 1 mM; and PMA, 100 nM. To calculate the normalized increase in PKD activity by the different stimuli, the appropriate basal value ($\pm Ca^{2-}$) was first subtracted and the values were normalized as listed in the Experimental section. $n = 4$ for all conditions. $^*P < 0.03$ compared with the control group.

[28]. The activities measured under these conditions were barely detectable and were a very small fraction ($\leqslant 3.0\%$, $n = 3$) of the basal activity measured in anti-PKD immunoprecipitates assayed using the βI peptide. This suggests that PKC proteins did not associate with PKD or were undetected under these conditions. Based on the kinase activities measured in anti-PKCδ and anti-PKCη immunoprecipitates assayed using either the βI or the ϵ substrate peptides, parotid acinar cells have a much larger amount of PKCδ activity compared with PKCη activity. (We did not measure other nPKC family members in this study). We also were unable to detect an association of PKD with cPKC and nPKC family members using Western blotting techniques (not shown). In addition, the immunoprecipitates that were used for the *in vitro* substrate-phosphorylation assays (Figure 4) were subjected to SDS/PAGE, and the dried gels were subjected to PhosphoImager analysis to analyse the resulting phosphoprotein pattern of proteins in the anti-PKD immunoprecipitates. Unlike similar studies reported by Waldron et al. [34], the only phosphoproteins identified were PKD ($\approx$ 110 kDa); proteins at the size of PKC family members ($\approx$ 80 kDa) were not observed. Thus, in freshly isolated rat parotid acinar cells that contain intrinsic levels of PKC proteins, we were unable to detect a PKC that co-associated with PKD (see Discussion).

Effects of Ca^{2+} and other conditions on PKD activity

Since the involvement of PKC in a P2X$_7$ receptor-initiated pathway appeared novel, we conducted additional experiments to determine more about the mechanism of action of ATP on PKD activity. Since ATP causes a decrease in the cell volume of rat parotid acinar cells due to the loss of intracellular ions [8], we exposed the cells to hypertonic conditions (the addition of 30 mM NaCl to the extracellular solution) that should promote a decreased cell volume. However, this did not increase the PKD activity (results not shown). ATP also produces a depolarization of the rat parotid acinar cells [15,20], but acute exposure of the cells to a depolarizing stimulus (the addition of 30 mM KCl to the extracellular solution) did not mimic the effects of ATP on PKD activity (results not shown). Thus, loss of cell volume and depolarization do not explain the activation of PKD by ATP.

ATP elevates intracellular Ca^{2+} by promoting an influx of Ca^{2+} through ATP-gated ion channels, and the removal of extracellular Ca prevents the ATP-stimulated elevation of $[Ca^{2+}]_i$. Although the Ca^{2+} ionophore ionomycin did not produce large changes in PKD activity (Figure 1, bottom panel), we investigated whether the removal of extracellular Ca^{2+} decreased the effects of ATP and other stimuli on PKD activity. The rationale for these experiments was that an elevation of $[Ca^{2+}]_i$ could be permissive for the activation of PKD by ATP. For these experiments, cells were suspended either in a solution containing 1 mM Ca^{2+} or in a Ca^{2+}-free solution containing 100 μM EGTA. The removal of extracellular Ca^{2+} had only a modest effect on the basal PKD activity, but the increases in PKD activity by ATP and carbachol were both reduced by $\approx 30\%$ in the absence of Ca^{2+} (Figure 5). The removal of extracellular Ca^{2+} did not significantly reduce the response to PMA. These results demonstrate that PKD activation by the P2X$_7$ receptor does not largely depend on Ca^{2+} entry. The activation of PKD by ATP and carbachol both had a small dependence on extracellular Ca^{2+}. As with the modest effects of ionomycin on PKD activity, these results suggest that a minor part of the activation of PKD is Ca^{2+}-sensitive.

Activation of P2X$_7$ receptors increases the tyrosine phosphorylation of PKCδ

Previously, we demonstrated that PKCδ activity in parotid acinar cells was increased by stimuli that increased PKCδ tyrosine phosphorylation, including muscarinic agonists and the phorbol ester PMA [6]. Since the studies of the activation of PKD by extracellular nucleotides suggested that these stimuli activate

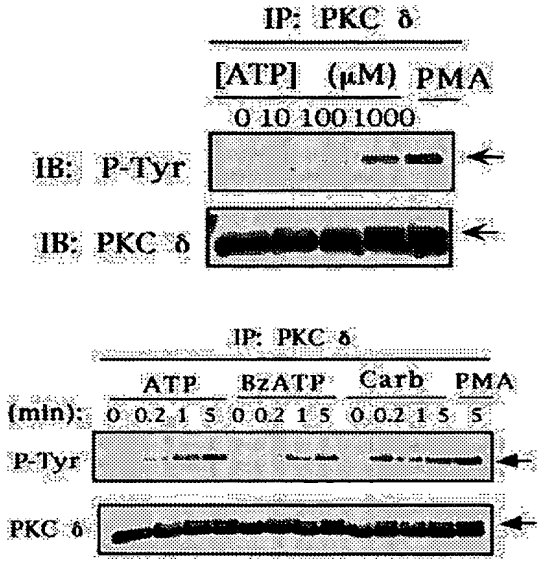

Figure 6 ATP, BzATP and other stimuli increase the tyrosine phosphorylation of PKCδ

Top panel: parotid acinar cells were exposed to 10–1000 μM ATP (2 min) or 100 nM PMA (5 min) and the cells were lysed, and PKCδ was immunoprecipitated using a monoclonal PKCδ-specific antibody. The immunoprecipitates were subjected to SDS/PAGE, transferred to nitrocellulose blotting paper, and sequentially immunoblotted (IB) using an anti-phosphotyrosine (P-Tyr) antibody and a polyclonal anti-PKCδ antibody. Bottom panel: parotid acinar cells were exposed to ATP (1 mM), BzATP (10 μM), carbachol (10 μM) and PMA (100 nM) for the indicated periods of time. PKCδ was immunoprecipitated and immunoblotted as in the top panel. Arrows in both panels indicate the location of the tyrosine-phosphorylated form of PKCδ.

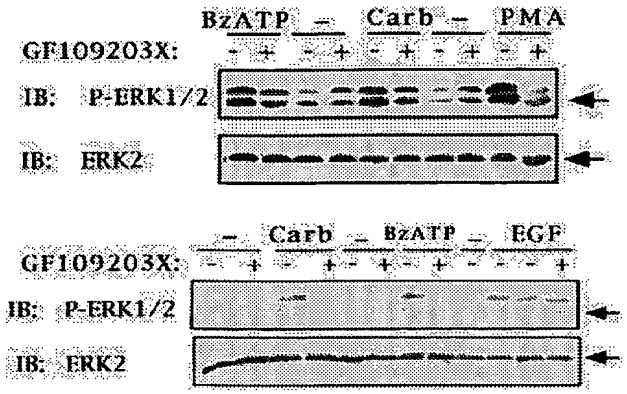

Figure 7 BzATP, carbachol and PMA, but not EGF, increase ERK1/2 activity in a PKC-dependent manner

Top panel: parotid acinar cells treated for 15 min with 3 μM GF109203X (+) or 0.06% DMSO (−) were exposed to BzATP (10 μM), carbachol (10 μM) and PMA (100 nM) for 2 min. Cells were lysed and the lysates were subjected to SDS/PAGE, transferred to nitrocellulose blotting paper and sequentially immunoblotted using phospho-ERK1/2 antibody and anti-ERK2 antibody. Bottom panel: cells treated with GF109203X (+) or DMSO (−) as in the top panel were exposed to carbachol (10 μM), BzATP (10 μM) and EGF (100 ng/ml) for 2 min. Cell lysates were immunoblotted as in the top panel. Arrows indicate the location of ERK2.

PKC, we examined the effects of P2X agonists on the tyrosine phosphorylation of PKCδ as one indicator of cellular PKC activity. High concentrations of ATP produced an increase in PKCδ tyrosine phosphorylation (Figure 6, top panel). Similar to the effects of ATP on PKD activation (Figure 1, second panel), 1 mM ATP was much more effective than lower concentrations. The time-dependence of alterations in PKCδ tyrosine phosphorylation by ATP, BzATP and carbachol is shown in Figure 6 (bottom panel). All three stimuli increased the tyrosine phosphorylation of PKCδ within 1 min, although carbachol produced relatively greater increases than those produced by ATP and BzATP at the earliest time point (0.2 min). Exposure of cells to ADP (1 mM) and UTP (1 mM) for 0.2–5 min did not appear to increase PKCδ tyrosine phosphorylation (results not shown). The effects of ATP and BzATP on PKCδ tyrosine phosphorylation were reduced in the presence of 5 mM extracellular Mg^{2+} (results not shown), consistent with these ligands acting via a $P2X_7$ receptor. BzATP (10 μM) also produced a measurable increase in PKCδ activity: a 2 min exposure to BzATP increased the activity by $12.5 \pm 0.7\%$ ($n = 3$) above basal levels.

These results indicate that PKCδ is stimulated by $P2X_7$ receptor agonists, but PKCδ may not be upstream of PKD activation. Although the tyrosine phosphorylation and activation of PKCδ are blocked by PP1 (an inhibitor of Src family members) and genistein (a general inhibitor of protein tyrosine kinases) [6,40], these inhibitors did not block the stimulatory effects of carbachol or ATP on PKD activation (Table 1). These results also suggest that the activation of Src and other tyrosine kinases does not play a major role in the activation of PKD by M3 muscarinic and $P2X_7$ receptors.

$P2X_7$ receptors initiate ERK1/2 activation in a PKC-dependent manner

The experiments presented above indicated that PKC and subsequently PKD were stimulated downstream of $P2X_7$ receptor activation. Therefore, we also examined the effect of nucleotide agonists on the MAP kinases ERK1 (p44 MAPK) and ERK2 (p42 MAPK), signalling proteins that are downstream of PKC activation in many cells, to determine if they represented other PKC-dependent signalling molecules that are affected by $P2X_7$ receptor stimulation. Exposure of parotid acinar cells to ATP and the $P2X_7$ agonist BzATP produced time-dependent increases in ERK1/2 activity, measured using a phospho-specific antibody that recognizes activated ERK1/2. Increases in ERK1/2 activity were observed within 1 min of exposure of cells to P2X and muscarinic agonists (results not shown). Increases in ERK1/2 activation by BzATP, carbachol and PMA (all at 2 min exposure) were reduced in cells pre-treated with 3 μM GF109203X, but the effects of EGF were not blocked by this PKC inhibitor (Figure 7). The stimulation of ERK1/2 in cells exposed to ATP (1 mM) was also blocked in cells treated with 3 μM GF109203X (not shown). These results are consistent with a PKC-mediated stimulation of p44/p42 MAPK upon activation of $P2X_7$ and muscarinic receptors and upon exposure of cells to PMA. The lack of an inhibitory effect of GF109203X on the activation of ERK1/2 by EGF is consistent with the ability of EGF to activate ERK1/2 in a PKC-independent manner in most cellular systems, and suggests that the inhibitory effects of GF109203X on ERK1/2 activation are due to its effects in blocking PKC upstream of ERK1/2. Thus, in addition to PKD, ERK1 and ERK2 are also PKC-dependent signalling proteins that are activated by both $P2X_7$ and muscarinic receptors in parotid acinar cells.

DISCUSSION

Our findings demonstrate that the activation of $P2X_7$ receptors, PLC-linked muscarinic and substance P receptors, and PMA promote an increase in both the activity and autophosphorylation of PKD in rat parotid acinar cells. These increases are sensitive to PKC inhibitors, suggesting that PKC is upstream of PKD activation by these stimuli. In addition, increases in PKD phosphorylation on Ser^{744}/Ser^{748} reflect the transphosphorylation of PKD downstream of PKC. The following data indicate that the effects of ATP on PKD were due mainly to the activation of a $P2X_7$ receptor: (1) high concentrations of ATP produced the largest increases in PKD activity (Figure 1, second panel); (2) extracellular Mg^{2+}, which reduces the potency of $P2X_7$ ligands, reduced the stimulatory effect of ATP (Table 1); (3) exposure of cells to DIDS, which blocks ATP binding to the $P2X_7$ receptor, reduced the stimulatory effect of ATP (Table 1); and (4) the $P2X_7$ ligand BzATP also activated PKD. These are all consistent with an effect of ATP on the $P2X_7$ receptors that are present on parotid acinar cells [14,17,20].

It is not surprising that muscarinic agonists stimulated PKD downstream of PKC, since a number of studies have demonstrated a role for PKC upstream of PKD [23,24], and muscarinic M3 receptor activation is known to lead to the stimulation of PKC. However, the PKC-dependent activation of PKD downstream of $P2X_7$ receptor activation was unexpected. Unlike the M3 muscarinic receptor, which is linked to PLCβ via its coupling to heterotrimeric G-proteins, receptors in the P2X family are not coupled to heterotrimeric G-proteins and do not share this coupling mechanism [9,10]. The similar effects of PKC inhibitors in reducing the activation of PKD by ATP, BzATP and carbachol (Table 1), indicate that the $P2X_7$ receptor and the M3 muscarinic receptor share a common mechanism (i.e. PKC activation) of PKD activation in rat parotid acinar cells. These results were very surprising, since the $P2X_7$ receptor is a ligand-gated non-selective cation channel, and to our knowledge its activation has not been reported to stimulate PKC.

The effects of BzATP and ATP on PKCδ tyrosine phosphorylation and activity and the GF109203X-sensitive stimulation of MAP kinases by ATP and BzATP also are observations consistent with the activation of PKC by the $P2X_7$ receptor/ion channel. The activation of other ion channels, including such ligand-gated ion channels as the nicotinic acetylcholine receptor, can activate PKC via the elevation of $[Ca^{2+}]_i$ [41,42]. However, although ionomycin had a modest stimulatory effect on PKD (Figure 1, bottom panel), alterations in $[Ca^{2+}]_i$ cannot fully account for the effects of ATP on PKD (Figures 1, bottom panel, and 5). Ionomycin also did not activate ERK1/2 in parotid acinar cells (not shown), indicating that the ATP/BzATP-initiated ERK1/2 activation was not due to the elevation of $[Ca^{2+}]_i$ through $P2X_7$ receptor/channel-mediated Ca^{2+} entry.

The results obtained using the PKC inhibitors suggest that members (or a member) of the cPKC or nPKC subtype of PKC family members are involved in mediating the effects of muscarinic and $P2X_7$ stimuli in activating PKD and ERK1/2. PKC proteins from these subgroups are inhibited by Ro31-8220 and GF109203X, although higher concentrations of the latter also block members of the atypical PKC group. Our data indicate that at least one nPKC family member, PKCδ, was activated by $P2X_7$ receptor agonists. The time courses of BzATP and ATP (Figure 6, bottom panel) and the concentration dependence of ATP (Figure 6, top panel) on PKCδ tyrosine phosphorylation, which we used as a reporter for the activation of this protein [6], were similar to the time course for the activation of PKD. However, these studies do not necessarily implicate PKCδ in the activation of PKD. PKD activation was not promoted by pervanadate, which increases PKCδ activity in parotid acinar cells [6], and was not blocked by genistein and PP1, which block stimulus-dependent PKCδ tyrosine phosphorylation and activity in parotid acinar cells [6,40]. This suggests that the stimulation of PKCδ may not be upstream of PKD activation, but this possibility will have to be confirmed in additional studies. These data also do not address the specific PKC family member(s) involved in the activation of ERK1/2 by $P2X_7$ and muscarinic receptors, although PKCδ has been implicated for some stimuli [43].

PKC proteins directly phosphorylate PKD on Ser^{744} and Ser^{748}, and these phosphorylations are required for PKD activation [25,33,44], consistent with earlier studies that demonstrated a PKC dependence [23,24]. This suggests that in some cells, at least part of the phosphorylation of PKD is due to its direct phosphorylation by one or several PKC proteins. In addition, PKD translocates to the plasma membrane in response to GPCR activation, and PKC proteins are required for the reverse of the translocation step [30]. Therefore, it is of interest that PKD can be co-immunoprecipitated with PKCη and to a limited extent with PKCϵ [24], and co-expression of PKCη and PKCϵ increased the activity of PKD [24,34]. Thus at least two members of the nPKC family appear to enhance the activity of PKD. PKCδ, another member of the nPKC family, was not examined in those studies, but PKCα (a cPKC) and ζ (an atypical PKC) did not associate with PKD.

We were unable to definitively detect the co-immunoprecipitation of another PKC with PKD in parotid cells. Although a very small amount of kinase activity was measured in anti-PKD immunoprecipitates when *in vitro* substrate-phosphorylation assays were performed using ϵ peptide, which is a poor PKD substrate but a good substrate for other PKCs, we cannot distinguish between a low level of a co-immunoprecipitated PKC versus a low efficiency of ϵ peptide phosphorylation by PKD. We also did not detect an $\approx$ 80 kDa protein that co-immunoprecipitated with PKD such that it could be identified when phosphorylation assays were conducted on anti-PKD immunoprecipitates (not shown). Finding such a protein at a size that was distinct from PKD ($\approx$ 110 kDa) led to the hypothesis and documentation that PKCη was co-immunoprecipitated with PKD in cells in which these proteins were overexpressed [34]. However, since our experiments were conducted using cells that express endogenous levels of proteins, this may have limited the detection of small amounts of associated proteins.

The time course of PKD activation by ATP (Figure 1, top panel) seemed to be slower than that produced by carbachol (Figure 1, third panel), although the effects of both carbachol and ATP were nearly maximal after a 1 min exposure of each stimulus. The relative magnitude of the increase in PKCδ tyrosine phosphorylation by carbachol after $\approx$ 15 s (Figure 6, bottom panel) appeared larger than those evoked by BzATP or ATP at the same time. These differences could be indicative of different post-receptor mechanisms that are used by the M3 receptor and the $P2X_7$ receptor to activate PKC. Potential mechanisms of PKC activation by $P2X_7$ receptors include phospholipase D, which can produce DAG and thereby activate PKC (for review, see [45]). Phospholipase D can be activated secondarily to elevations in $[Ca^{2+}]_i$ in rat parotid acini [46]. However, if phospholipase D is involved in the activation of PKD by ATP/BzATP in parotid acinar cells, it appears unlikely that the elevation of $[Ca^{2+}]_i$ is involved, since changes in $[Ca^{2+}]_i$ did not account for the effects of these stimuli on PKD activation.

The activation of the $P2X_7$ receptor was reported to produce modest increases in inositol phosphate levels in rat parotid acinar

cells, suggesting that high concentrations of ATP can produce some degree of activation of PLC [21]. Either there is some degree of crosstalk between the $P2X_7$ receptor and PLCβ or another PLC upstream of PKC activation, or else the $P2X_7$ receptor has another mechanism by which it activates members (or a member) of the PKC family. In fact, recently the $P2X_7$ receptor was detected to form a signalling complex with 11 distinct proteins, including one protein (phosphatidylinositol 4-kinase) which could lead to the production of lipids required for diacylglycerol generation [31]. In addition, the $P2X_7$ receptor itself was found to be phosphorylated on tyrosine residues, and its activation by BzATP resulted in its dephosphorylation, perhaps by receptor tyrosine phosphatase-β, which also associates with the $P2X_7$ receptor [31]. Thus the activation of this receptor may initiate numerous signalling events that are not typically associated with the opening of a non-selective cation channel, including kinase and phosphatase activation, and these may explain the $P2X_7$-promoted activation of PKC shown in the present studies.

We cannot yet address the physiological importance of PKD activation in parotid acinar cells, and the biological role of PKD is still unclear in many cellular systems. The substrate specificity of PKD is different from PKC family members [26–28]. PKD has been reported to regulate protein secretion at the Golgi membrane, where PKD affects sulphate uptake and glycosaminogylcan sulphation [29]. PKD/PKCμ was shown to mediate the phosphorylation of the EGF receptor on two threonine residues (Thr^{654} and Thr^{669}), and these phosphorylations blocked the EGF receptor-dependent activation of c-Jun N-terminal kinase [47]. These results indicate that the EGF receptor is downstream of PKD in some cell systems, and that this enzyme can play a critical role in signalling. PKD can also stimulate ERK1/2 by activating Raf-1 [48], and ERK1/2 activation can result from the phosphorylation and activation of PKD by PKC [49]. Thus, it may be possible that part of the activation of ERK1/2 by the $P2X_7$ receptor and muscarinic receptor may result from the activation of PKD by these receptors. On the basis of its association with phosphatidylinositol 4-kinase and phosphatidylinositol-4-phosphate 5-kinase, it was suggested that PKD could act as a scaffold for lipid kinases [36]. Therefore, the stimulation of PKD by the $P2X_7$ receptor (the present study) and the association of phosphatidylinositol 4-kinase with both the $P2X_7$ receptor [31] and PKD [36] raises the possibility that there also may be a close association of PKD with the $P2X_7$ receptor.

In summary, our studies demonstrate that PKD and ERK1/2 are activated in rat parotid acinar cells by $P2X_7$ receptors as well as by GPCRs. The pathways to the activation of PKD and ERK1/2 by both kinds of receptors depend on PKC and do not primarily depend on the elevation of $[Ca^{2+}]_i$. Thus, PKD, PKCδ and the MAP kinases ERK1 and ERK2 are signalling proteins that are downstream of diverse types of receptors, and the stimulation of these signalling proteins is an indicator for the activation of PKC by the $P2X_7$ receptor.

We thank Yue Zheng for excellent technical assistance, and Cyril Benes, Lewis C. Cantley and Kiyotaka Nishikawa for helpful conversations. This work was supported in part by National Institutes of Health grant DE10877 (to S.P.S.).

REFERENCES

1 Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C.-S., Newton, A. C., Schaaffhausen, B. S. and Toker, A. (1998) Regulation of protein kinase C ζ by PI 3-kinase and PKD-1. Curr. Biol. **8**, 1069–1077

2 Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. **9**, 484–496

3 Hug, H. and Sarre, T. F. (1993) Protein kinase C isoenzymes: divergence in signal transduction? Biochem. J. **291**, 329–343

4 Newton, A. (1995) Protein kinase C: structure, function, and regulation. J. Biol. Chem. **270**, 28495–28498

5 Soltoff, S. P. and Toker, A. (1995) Carbachol, substance P, and phorbol ester promote the tyrosine phosphorylation of protein kinase Cδ in salivary gland epithelial cells. J. Biol. Chem. **270**, 13490–13495

6 Benes, C. and Soltoff, S. P. (2001) Modulation of PKCδ tyrosine phosphorylation and activity in salivary and PC12 cells by src -related kinases. Am. J. Physiol. **280**, C1498–C1510

7 McMillian, M. K., Soltoff, S. P., Lechleiter, J. D., Cantley, L. C. and Talamo, B. R. (1988) Extracellular ATP increases free cytosolic calcium in rat parotid acinar cells. Biochem. J. **255**, 291–300

8 Soltoff, S. P., McMillian, M. K., Cragoe, Jr, E. J., Cantley, L. C. and Talamo, B. R. (1990) Effects of extracellular ATP on ion transport systems and $[Ca^{2-}]_i$ in rat parotid acinar cells. Comparison with the muscarinic agonist carbachol. J. Gen. Physiol. **95**, 319–346

9 Abbracchio, M. P. and Burnstock, G. S. (1995) Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol. Ther. **64**, 445–475

10 Boarder, M. R. and Hourani, S. M. O. (1998) The regulation of vascular function by P2 receptors: multiple sites and multiple receptors. Trends Pharmacol. Sci. **19**, 99–107

11 Surprenant, A., Buell, G. and North, R. A. (1995) P2X receptors bring new structure to ligand-gated ion channels. Trends Neurosci. **18**, 224–229

12 Norenberg, W. and Illes, P. (2000) Neuronal P2X receptors: localisation and functional properties. Naunyn-Schmiedeberg's Arch. Pharmacol. **362**, 324–339

13 Soltoff, S. P., McMillian, M. K., Lechleiter, J. D., Cantley, L. C. and Talamo, B. R. (1990) Elevation of $[Ca^{2-}]i$ and the activation of ion channels and fluxes by extracellular ATP and phospholipase C-linked agonists in rat parotid acinar cells. Ann. N.Y. Acad. Sci. **603**, 76–92

14 McMillian, M. K., Soltoff, S. P., Cantley, L. C., Rudel, R. A. and Talamo, B. R. (1993) Two distinct cytosolic calcium responses to extracellular ATP in rat parotid acinar cells. Br. J. Pharmacol. **108**, 453–461

15 Tenneti, L. and Talamo, B. R. (1993) Modulation of extracellular ATP-induced Ca^{2-} responses: role of protein kinases. Biochem. J. **295**, 255–261

16 Turner, J. T., Landon, L. A., Gibbons, S. J. and Talamo, B. R. (1999) Salivary gland P2 nucleotide receptors. Crit. Rev. Oral Biol. Med. **10**, 210–224

17 Tenneti, L., Gibbons, S. J. and Talamo, B. R. (1998) Expression and trans-synaptic regulation of P_{2X4} and P_{2Z} receptors for extracellular ATP in parotid acinar cells. J. Biol. Chem. **273**, 26799–26808

18 Surprenant, A., Rassendren, F., Kawashima, E., North, R. A. and Buell, G. (1996) The cytolytic P_{2Z} receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). Science **272**, 735–738

19 Dubyak, G. R. and El-Moatassim, C. (1993) Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucleotides. Am. J. Physiol. **265**, C577–C606

20 Soltoff, S. P., McMillian, M. K. and Talamo, B. R. (1992) ATP activates a cation-permeable pathway in rat parotid acinar cells. Am. J. Physiol. **262**, C934–C940

21 Jorgensen, T. D., Gromada, J., Tritsaris, K., Nauntofte, B. and Dissing, S. (1995) Activation of P_{2Z} receptors diminishes the muscarinic cholinergic-induced release of inositol 1,4,5-trisphosphate and stored calcium in rat parotid acini. Biochem. J. **312**, 457–464

22 Lee, M. G., Zeng, W. and Muallem, S. (1997) Characterization and localization of P2 receptors in rat submandibular gland acinar and duct cells. J. Biol. Chem. **272**, 32951–32955

23 Zugaza, J. L., Waldron, R. T., Sinnett-Smith, J. and Rozengurt, E. (1997) Bombesin, vasopressin, endothelin, bradykinin, and platelet-derived growth factor rapidly activate protein kinase D through a protein kinase C-dependent signal transduction pathway. J. Biol. Chem. **272**, 23952–23960

24 Zugaza, J. L., Sinnett-Smith, J., Van Lint, J. and Rozengurt, E. (1996) Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. EMBO J. **15**, 6220–6230

25 Waldron, R. T., Iglesias, T. and Rozengurt, E. (1999) Phosphorylation-dependent protein kinase D activation. Electrophoresis **20**, 382–390

26 Valverde, A. M., Sinnett-Smith, J., Van Lint, J. and Rozengurt, E. (1994) Molecular cloning and characterization of protein kinase D: a target for diacylglycerol and phorbol esters with a distinctive catalytic domain. Proc. Natl. Acad. Sci. U.S.A. **91**, 8572–8576

27 Van Lint, J., Sinnett-Smith, J. and Rozengurt, E. (1995) Expression and characterization of PKD, a phorbol ester and diacylglycerol-stimulated serine protein kinase. J. Biol. Chem. **270**, 1455–1461

28 Nishikawa, K., Toker, A., Johannes, F.-J., Songyang, Z. and Cantley, L. C. (1997) Determination of the specific substrate sequence motifs of protein kinase C isozymes. J. Biol. Chem. **272**, 952–960

29 Prestle, J., Pfizenmaier, K., Brenner, J. and Johannes, F.-J. (1996) Protein kinase C μ is located at the golgi compartment. J. Cell Biol. **134**, 1–10
30 Rey, O., Young, S. H., Cantrell, D. and Rozengurt, E. (2001) Rapid protein kinase D translocation in response to G protein-coupled receptor activation. J. Biol. Chem. **276**, 32616–32626
31 Kim, M., Jiang, L.-H., Wilson, H. L., North, R. A. and Surprenant, A. (2001) Proteomic and functional evidence for a $P2X_7$ receptor signaling complex. EMBO J. **20**, 6347–6358
32 Matthews, S. A., Rozengurt, E. and Cantrell, D. (1999) Characterization of serine 916 as an *in vivo* autophosphorylation site for protein kinase D/protein kinase Cμ. J. Biol. Chem. **274**, 26543–26549
33 Igleisas, T., Waldron, R. T. and Rozengurt, E. (1998) Identification of *in vivo* phosphorylation sites required for protein kinase D activation. J. Biol. Chem. **273**, 27662–27667
34 Waldron, R. T., Iglesias, T. and Rozengurt, E. (1999) The plextrin homology domain of protein kinase D interacts preferentially with the η isoform of protein kinase C. J. Biol. Chem. **274**, 9224–9230
35 Hausser, A., Storz, P., Link, G., Stoll, H., Liu, Y.-C., Altman, A., Pfizenmaier, K. and Johannnes, F.-J. (1999) Protein kinase μ is negatively regulated by 14-3-3 signal transduction proteins. J. Biol. Chem. **274**, 9258–9264
36 Nishikawa, K., Toker, A., Wong, K., Marignani, P. A., Johannes, F.-J. and Cantley, L. C. (1998) Association of protein kinase Cμ with type II phosphatidylinositol 4-kinase and type I phosphatidylinositol-4-phosphate 5-kinase. J. Biol. Chem. **273**, 23126–23133
37 Soltoff, S. P. (2001) Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase Cδ tyrosine phosphorylation. J. Biol. Chem. **276**, 37986–37992
38 Soltoff, S. P., McMillian, M. K., Talamo, B. R. and Cantley, L. C. (1993) Blockade of ATP binding site of P2 purinoceptors in rat parotid acinar cells by isothiocyanate compounds. Biochem. Pharm. **45**, 1936–1940
39 Matthews, S. A., Pettit, G. R. and Rozengurt, E. (1997) Bryostatin 1 induces biphasic activation of protein kinase D in intact cells. J. Biol. Chem. **272**, 20245–20250
40 Bradford, M. D. and Soltoff, S. P. (1998) Involvement of protein kinases and phosphatases in tyrosine phosphorylation of PKCδ in rat parotid acinar cells exposed to secretory stimuli. Eur. J. Morph. **36**, 176–180
41 Nishizaki, T. and Sumikawa, K. (1998) Nicotinic receptors are regulated by protein kinase C activated via a nicotinic receptor-mediated signaling pathway. Brain Res. Mol. Brain Res. **61**, 211–218
42 Swope, S. L., Moss, S. I., Raymond, L. A. and Huganir, R. L. (1999) Regulation of ligand-gated ion channels by protein phosphorylation. Adv. Sec. Mes. Phosphoprot. Res. **33**, 49–78
43 MacKenzie, S., Fleming, I., Houslay, M. D., Anderson, N. G. and Kilgour, E. (1997) Growth hormone and phorbol esters require specific protein kinase C isoforms to activate mitogen-activated protein kinases in 3T3-F442A cells. Biochem. J. **324**, 156–165
44 Waldron, R. T., Rey, O., Iglesias, T., Tugal, T., Cantrell, D. and Rozengurt, E. (2001) Activation loop Ser^{744} and Ser^{748} in protein kinase D are transphosphorylated *in vivo*. J. Biol. Chem. **276**, 32606–32615
45 Liscovitch, M. and Chalifa, V. (1994) Signal-activated phospholipase D. In Signal-Activated Phospholipases (Liscovitch, M., ed.), pp. 31–63, R.G. Landes Company, Austin, TX
46 Guillemain, I. and Rossignol, B. (1994) Receptor- and phorbol ester-mediated phospholipase D activation in rat parotid involves two different pathways. Am. J. Physiol. **266**, C692–C699
47 Bagowski, C. P., Stein-Gerlach, M., Choidas, A. and Ullrich, A. (1999) Cell-type specific phosphorylation of threonines T654 and T996 by PKD defines the signal capacity of the EGF receptor. EMBO J. **18**, 5567–5576
48 Hausser, A., Storz, P., Hubner, S., Braendlin, I., Martinez-Moya, M., Link, G. and Johannes, F.-J. (2001) Protein kinase C μ selectively activates the mitogen-activated protein kinase (MAPK) p42 pathway. FEBS Lett. **492**, 39–44
49 Brandlin, I., Hubner, S., Eiseler, T., Martinez-Moya, M., Horschinek, A., Hausser, A., Link, G., Rupp, S., Storz, P., Pfizenmaier, K. and Johannes, F.-J. (2002) PKCη mediated PKCm-activation modulates ERK and JNK signal pathways. J. Biol. Chem. **277**, 6490–6496

Received 1 March 2002/3 June 2002; accepted 11 June 2002
Published as BJ Immediate Publication 11 June 2002, DOI 10.1042/BJ20020358